



Isolation, Identification, and Antimicrobial Study of *Yersinia* spp. Isolated from Traditional and Industrial Olivier Salad Specimens in Tehran

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ABSTRACT

Background: *Yersinia enterocolitica* is a gram-negative bacteria and one of the most important food-borne bacteria that causes yersiniosis. Several studies have investigated various species of this gastrointestinal pathogen in food and water sources. Although Olivier salad is a ready-to-eat and easily available cold food, it is highly susceptible to microbial contaminations. This study aims to investigate the presence of *Yersinia* spp., especially the pathogenic *Yersinia enterocolitica* in Olivier salad. **Methods:** In this cross-sectional descriptive study, 150 specimens (traditional and industrial Olivier salad) were collected and tested for the presence of *Yersinia* spp. Using the Iranian National Standard No. 4556. Obtained data were analyzed statistically using descriptive statistics in IBM SPSS. **Results:** *Yersinia* spp. contamination was found in only 6% of traditional specimens (6 out of 100). Based on biochemical reactions, four isolates of *Yersinia enterocolitica* and two isolates of *Yersinia intermedia* were identified from six *Yersinia* isolates. The biotype of *Yersinia enterocolitica* was investigated, and three strains of biotype 1A and one strain of biotype 1B were identified. The human pathogenic serotype was not found in the serotype analysis. **Conclusion:** The study results showed that specimens of traditionally prepared Olivier salad were contaminated with *Yersinia* spp. *Yersinia enterocolitica* is the most prevalent species as expected, and the analysis of the strains of this species revealed that it also contains other biotypes, including the highly pathogenic biotype 1B.

Introduction

Yersinia enterocolitica (YE), a gram-negative, oxidase-negative, and facultative anaerobic species, is highly heterogeneous with several biotypes and serotypes (Grant *et al.*, 1998). A few of whom have been associated with human disease. The majority of YE strains associated with human

Yersiniosis are Bioserotypes (1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, 4/O:3) (Shoab *et al.*, 2019). *Yersinia* contamination is a major issue in food supplies because this bacterium takes a long time to grow (Fredriksson-Ahomaa and Korkeala, 2003). YE is a psychotropic enteropathogen found in food and

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water. Since this microorganism can multiply enormously at refrigeration temperatures, meat, chicken, milk, and cheese contaminated with this species may pose serious health risks to consumers (Sharifi Yazdi *et al.*, 2023). This microorganism is typically a gastrointestinal pathogen with a strong proclivity for additional intestinal unfold under specific host conditions (Soltan Dallal and Moezardalan, 2004). In immune-compromised patients, systemic and extraintestinal infections and enterocolitis necessitate antibiotic therapy, with the most commonly used agents being chloramphenicol, gentamicin, tetracycline, cotrimoxazole, and ciprofloxacin (Fàbrega and Vila, 2012). The emergence of yersiniosis can be probably attributed to differences in livestock husbandry, food technology, and the food industry. While many modern slaughter techniques reduce the risk of meat contamination, possibilities for organism transmission from animal to animal and cross-contamination of carcasses and meat merchandise exist on a scale that was not previously recognized. Furthermore, advancements in packaging and refrigeration now allow industry and consumers to keep meals for much longer periods, which is important for a cold-adapted pathogen such as YE (Bari *et al.*, 2011).

The use of ready-to-eat foods, such as salads, has increased due to lifestyle changes, the increased urbanization rate, and the benefits of such foods, such as their ease of use and ability to be prepared quickly. Iran is a developing country where these food products, particularly Olivier salad, are widely consumed. Because of the variety of Olivier salad ingredients and its preparation process, which involves manual interference (e.g., cutting or crushing), it is highly susceptible to contamination. The cold chain is considered to be the only way to control the quality of RTE Olivier salads after they are produced. However, some pathogenic psychotropic bacteria, such as YE, can grow at cold temperatures (Ercolini *et al.*, 2011). Therefore, this study investigated the biotypes and serotypes as well as the antibiotic resistance pattern of *Yersinia* spp. isolated from traditional and industrial Olivier salad specimens marketed in

Tehran. Rich in essential nutrients, Olivier salad is particularly vulnerable to microorganisms contributing to food spoilage, necessitating appropriate packaging solutions. Active packaging is characterized as a system composed of dynamic polymers and supplementary components. Active antimicrobial packaging systems composed of nanocomposites represent an innovative category of nanostructured packaging generated through the direct integration of antimicrobial nanoparticles with a polymeric matrix. These systems possess the capability to eradicate or inhibit the proliferation of pathogenic microorganisms present in food products (Valipour Motlagh *et al.*, 2021).

Materials and Methods

Sample collection

In this study, random sampling was carried out from January 2021 to July 2021 (for six months) in order to investigate YE in the industrial and traditional Olivier salad. Accordingly, 100 specimens of traditional Olivier salad and 50 specimens of industrial salads were gathered and prepared.

Cold enrichment, bacterial isolation, and identification

For the microbial detection of YE from traditional and industrial Olivier salad specimens, 10 grams of each specimen was added to 90 ml of Tryptic Soy Broth-Yeast Extract (TSB-YE) pre-enrichment medium under completely sterile conditions which were incubated at 30 °C for 24 hours. After the incubation period was finished, 10 ml of the prepared suspension was poured into a new sterile container. It was then uniformly topped off with 90 ml of Peptone Sorbitol Bile Broth (PSB) enrichment medium and refrigerated at 4 °C for 7 to 15 days. A loop of this mixture was then cultured linearly on Cefsulodin Irgasan Novobiocin Agar (CIN) with *Yersinia* selective supplement every week using 0.5 ml of the enriched suspension and 4.5 ml of an alkaline potassium hydroxide solution (0.5% KOH in 0.5% NaCl). Cultured plates were incubated at 25 °C for 24–48 hours (Heras-Saizarbitoria and Boiral, 2013). Then, the colonies were examined macroscopically and microscopically. Suspect colonies (small and

smooth with a red center and translucent rim or red bull's-eye-like colonies) were tested for motility in SIM medium at 25 and 37 °C. To identify the bacteria, biochemical tests such as urease activity, Kligler Iron Agar (KIA) behavior, Simon citrate, Lysine decarboxylase, and ornithine decarboxylase were also used. The identification tests of *Yersinia* isolates from the strains identified for *Yersinia* bacteria were also completed by Oxidase test, ONPG test, and API (Analytical Profile Index) 20E test (bioMérieux France) (Soltan Dallal and Moezardalan, 2004). Identifying the biotype and serotype of *Yersinia* isolates, this study considered the isolation and identification of *Yersinia* species in the Olivier salad specimens, particularly its pathogenic species. The specimens were also subjected to biochemical tests (*i.e.*, fermentation of xylose, trehalose, rhamnose, squalene, search for indole, tween esterase, and pyrazinamide) in order to determine their biotype. As a result, the biotypes were determined based on YE reaction culture media and its compatibility with standard sources. According to biochemical properties, YE strains are classified into six biotypes (1A, 1B, 2, 3, 4, and 5). **Table 1** presents the responses of the various biotypes to various tests (Soltan Dallal and Moezardalan, 2004).

Table 1. Biochemical tests needed for biotyping *Yersinia*.

Test	1A	1B	2	3	4	5
Salicin (acid production in 24 H)	+	-	-	-	-	-
Esculin (24 H)	+/-	-	-	-	-	-
Xyloza (acid production)	+	+	+	+	-	V
Trehaloza (acid production)	+	+	+	+	+	-
Indole production	+	+	V	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+
Inositol (acid production)	+	+	+	+	+	+
Sorbose (acid production)	+	+	+	+	+	-
pyrazinamidase	+	-	-	-	-	-
lipase activity	+	+	-	-	-	-

V: Variable

Serotyping was performed on selected strains with typical characteristics using commercial antisera O:3, O:5, O:8, and O:9 (MAST URI®).

The isolated colonies were suspended in a physiological solution. The suspensions were then treated with a drop of antiserum specific to the individual pathogenic serotypes O:3, O:5,27, O:8, or O:9 (Bari *et al.*, 2011).

Antimicrobial susceptibility

The agar diffusion method was used to test the susceptibility of bacterial strains on Mueller-Hinton agar at 28 °C. The antibiotic sensitivity spectrum of each isolate was determined using the CLSI protocol (Clinical and Laboratory Standard Institute (CLSI), 2023). Antibacterial agents included cefotaxime 30 mcg (CTX), ceftriaxone 30 mcg (CRO), ciprofloxacin 5 mcg (CP), trimethoprim-sulfamethoxazole 25 mcg (SXT), chloramphenicol 30 mcg, ampicillin 10 mcg (AMX), nalidixic acid 30 mcg (NA), and tetracycline 30 mcg (TE). Antibiotic disks were provided from Mast Company.

Ethics considerations

This study resulted from a research grant approved by the Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran, with the ethics code IR.TUMS.SPH.REC.1399.207.

Data analysis

The research data were analyzed statistically using Chi-square test and independent t-test in SPSS-18.

Results

Yersinia spp. sampling and isolation

This study was carried out on 150 samples of Olivier salad, including 50.0 samples from 5 brands offered in the regions under investigation of industrial Olivier salad and 100.0 samples of traditional salad. These samples were collected from the production and supply centers in the areas covered by Tehran University of Medical Sciences with two types of protein (chicken or sausage). 80 samples of Olivier salad had chicken and 20 samples had sausage. In connection with industrial Olivier salad, 38 samples had chicken and 12 samples had sausage.

Identification of *Yersinia* spp., biotypes, and

serotypes

Figure 1 YE and *Yersinia intermedia* were discovered in contaminated specimens at a 2:1 ratio using differential media and the API-20E kit. In this study, four YE isolates from traditional Olivier salad belonged to biotype 1, three of which were biotype 1A and one was biotype 1B. As expected, the most common *Yersinia* spp. in this study was YE, with the highest percentage of biotype 1A. However, the presence of the highly pathogenic biotype 1B can be alarming. Specific antiserum of human pathogenic serotypes O:8 and O:5 were used to determine the serotype of YE for 1B and 1A biotypes. Agglutination was not observed when the isolate was exposed to the specific antiserum, indicating that the human pathogenic strains of YE (1B/O:8 and 1A/O:5) were not detected in specimens, and the strains are most likely isolated from the environment or contain an animal pathogen.

Antimicrobial studies

All isolates of YE biotype 1B and isolates of YE

biotype 1A were sensitive to cefotaxime, ceftriaxone, ciprofloxacin, trimethoprim-sulfamethoxazole, nalidixic acid, and chloramphenicol, but YE biotype 1B was resistant to ampicillin (one specimen) and relatively sensitive to tetracycline antibiotic (75%). In addition, all isolates of *Yersinia intermedia* were sensitive to cefotaxime (100%), ceftriaxone (100%), ciprofloxacin (100%), trimethoprim-sulfamethoxazole (100%), nalidixic-acid (100%), chloramphenicol (100%), ampicillin (75%), and tetracycline (25%). Briefly, the Disk Diffusion method and antibiotic disks (containing cefotaxime 30 mg, ceftriaxone 30 mg, ciprofloxacin 5 mg, trimethoprim-sulfamethoxazole 25 mg, chloramphenicol 30 mg, ampicillin 10 mg, nalidixic acid 30 mg, and tetracycline 30 mg) were used to conduct an antimicrobial test to determine the sensitivity and drug resistance pattern of YE.

Microbial count

Table 2 presents the results on total microbial count in various specimens of traditional and industrial Olivier salad.

Table 2. The count and percentage of acceptable and unacceptable specimens of traditional and industrial Olivier salad based on the total microbial count per gram of food (CFU/g), according to the Iranian National Standard No. 4556 permissible limit.

Industrial olivier salad (50 samples)		Traditional olivier salad (100 samples)	
Acceptable	Unacceptable	Acceptable	Unacceptable
38 (76%)	12 (24%)	3 (3%)	97 (97%)

The overall level of microbial contamination (unacceptable specimens) in traditional Olivier salad was significantly higher than in industrial Olivier salad, as shown in the table. As a result, there may be

a direct relationship between the severity of contamination and the type of Olivier salad in terms of traditional or industrial preparation methods, which ultimately affects the product's acceptability.



Figure 1. A) *Yersinia intermedia* and B) *Yersinia enterocolitica* biochemical test results.

Meat source of Olivier salad

When it comes to the meat source used to prepare Olivier salad, there are two options in Tehran market: chicken-based and sausage-based. Table 3 presents

the percentage of *Yersinia* bacteria in Olivier salad specimens containing chicken which was 7.5%, whereas there were no contaminations in Olivier salad specimens containing sausage.

Table 3. The frequency of *Yersinia* spp. in traditional and industrial Olivier salad according to the type of protein material.

Traditional Olivier salad (100)				Industrial Olivier salad (50)			
Chicken-based (80)		Sausages-based (20)		Chicken-based (38)		Sausages-based (12)	
n	%	n	%	n	%	n	%
6.0	7.5	0.0	0.0	0.0	0.0	0.0	0.0

The results indicated that Olivier salad specimens containing chicken were more contaminated than those containing sausage. It can be hence concluded that there is a relationship between the intensity of contamination and the type of protein source (chicken or sausage) of Olivier salad.

Figure 2 shows the results based on a two-way

analysis the amount of *Yersinia* spp. in Olivier salad containing chicken was significantly ($P<0.0001$) higher than the Olivier salad containing sausage in the traditional Olivier salad, so it can be concluded that there was a relationship between the presence of *Yersinia* spp and the type of protein material.

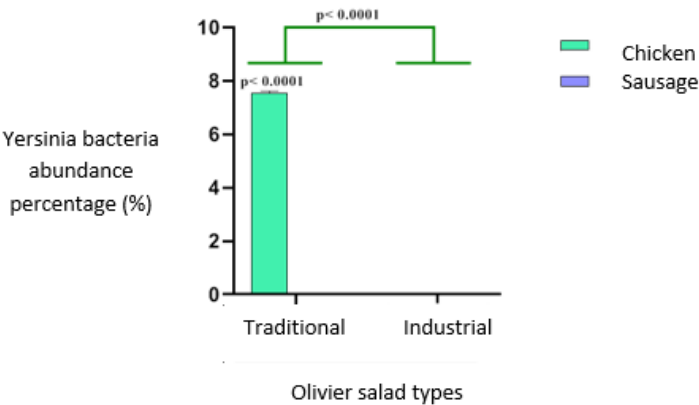


Figure 2. Evaluating the percentage of *Yersinia* spp. in traditional and industrial Olivier salad according to the type of protein raw material (chicken and sausage), with the help of two-way analysis of variance statistical method in GraphPad Prism software (Mean \pm SD).

However, Table 4 a comparison of the frequency percentage of acceptable specimens of industrial chicken-based (73%), industrial sausage-based (83%), and traditional chicken-based (1.25%)

specimens showed that among the traditional chicken and sausage specimens, the proportion of acceptable specimens containing sausage was higher than that of chicken in both groups.

Table 4. Frequency percentage of acceptable and unacceptable samples in terms of total microbial count (CFU/gr) in traditional and industrial Olivier salad containing chicken and sausage.

Traditional Olivier salad (100)				Industrial Olivier salad (50)				Total Olivier salad (150)			
Chicken-based		Sausage-based		Chicken-based		Sausage-based		Chicken-based		Sausage-based	
Ac	Unac	Ac	Unac	Ac	Unac	Ac	Unac	Ac	Unac	Ac	Unac
1 ^a	79	2	18	28	10	10	2	29	89	11	21
(1.2)	(98.7)	(10.0)	(90.0)	(73.0)	(26.4)	(83.4)	(16.6)	(24.0)	(75.4)	(35.0)	(65.0)

^a: n (%); Ac: Accetable; Unac: Unaccetable.

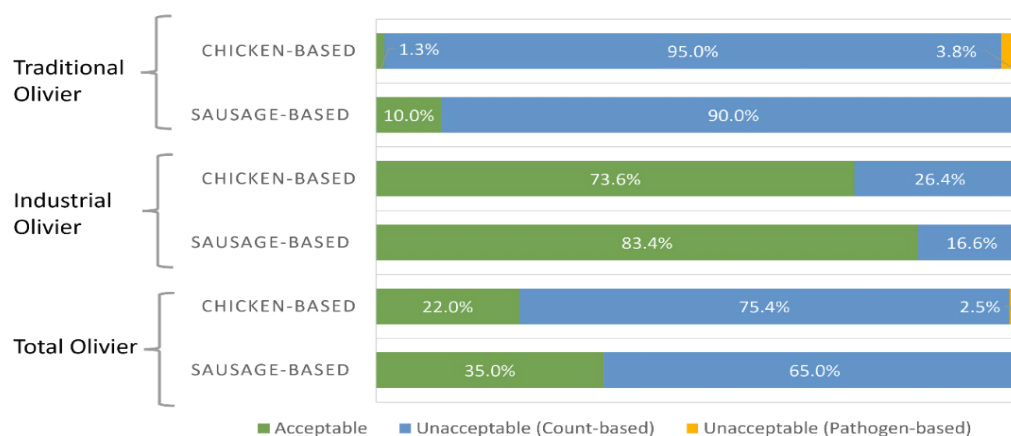
Investigation of pathogenic and non-pathogenic microbial contamination

Olivier salad specimens were examined for the presence of pathogenic bacteria. According to **Table 5**, the number of pathogenic bacteria, such as YE, *Escherichia coli*, *Shigella* spp., and *Salmonella*, spp. was significantly higher in the traditional specimens of Olivier salad when compared to industrially prepared Olivier salad. The frequency of the total non-pathogenic bacteria identified (in the total number of infected samples and the number of unacceptable infected samples) was higher in

traditional type compared with the industrial one. Meanwhile, the results of the table showed that the frequency of non-pathogenic bacteria in the total number of infected samples (acceptable and unacceptable) was different from unacceptable infected samples. Based on **Figure 3**, the reason is that, although non-pathogenic bacteria are detected in the sample which are considered contamination, since the number obtained from the overall microbial count of the sample was in accordance with the number of the standard limit (maximum 10⁵), the sample counts of Microbial was acceptable.

Table 5. The number and percentage of identified pathogens in all samples of traditional and industrial Olivier salad.

Bacterial genus	Traditional Olivier salad (100)		Industrial Olivier salad (50)	
	n	%	n	%
<i>Yersinia enterocolitica</i>	4.0	4.0	0.0	0.0
<i>Escherichia coli</i>	3.0	3.0	1.0	2.0
<i>Shigella</i> spp.	1.0	1.0	0.0	0.0
<i>Salmonella</i> spp.	1.0	1.0	0.0	0.0

**Figure 3.** The overview of acceptable and unacceptable specimens regarding the meat source and the reason for rejection (count-based or both count and pathogen-based).

In this chart, the number of specimens for each row is 80 for chicken-based traditional Olivier, 20 for sausage-based traditional Olivier, 38 for chicken-based industrial Olivier, 12 for sausage-based industrial Olivier, 118 for chicken-based total Olivier samples, and 32 for sausage-based total Olivier samples.

Discussion

Yersinia enterocolitica is one of the 5 pathogenic bacteria in the digestive system, and infection with this bacterium frequently results in enterocolitis (Koushki, 2023, Soltan Dallal *et al.*, 2006). Although the symptoms are usually self-limiting abdominal pain, extra-intestinal forms such as spleen abscesses, cholecystitis, and septicemia have been associated with 50% of mortalities in immunocompromised patients (Lorenzo *et al.*, 2018, Wang *et al.*, 2019). As a foodborne pathogen, this bacterium is now important for humans (Bancerz-Kisiel and Szweda, 2015).

On the other hand, only a few of the 60-70 known serotypes are involved in causing disease in humans. It is hence critical to distinguish pathogenic strains from non-pathogenic ones in order to control this pathogen in food (Estrada *et al.*, 2020, Soltan Dallal *et al.*, 2017). Today, as a result of changes in people's eating habits and lifestyles, there is a greater desire to consume cold, prepared foods like salads. Consequently, the risk of disease transmission from all types of bacteria has increased, especially from cold-resistant bacteria such as the food-borne *Yersinia* spp (Patel and Rathod, 2017). Therefore, this study examined the presence of *Yersinia* bacteria, identified its species, particularly the pathogenic species, and determined the biotypes and serotypes as well as the pattern of antibiotic resistance of *Yersinia* isolates in traditional and industrial Olivier salad specimens. To this end, 50 specimens of industrial Olivier salad sold in stores in Tehran and 100 specimens of traditionally prepared Olivier salad sold in salad preparation and distribution centers were tested for the presence of *Yersinia* bacteria. Traditional specimens had a 6% contamination

level (6 out of 100), industrial specimens had no contamination (0%), and all 150 salad specimens had a 4% contamination level (6 out of 150). Out of 6 *Yersinia* isolates, 4 isolates were determined to be YE and 2 isolates were *Yersinia intermedia* based on biochemical reactions. In a similar study (Söderqvist *et al.*, 2016), collected 141 specimens of packaged ready-to-eat salads, containing cooked chicken, ham, and vegetables, from the refrigerators of salad preparation and supply centers. According to the findings, YE bacteria infected 7 (5%) of the salad specimens. Furthermore, keeping food at refrigerator temperature (4-8 °C) was thought to promote the growth of cold-oriented bacteria such as YE (Söderqvist *et al.*, 2016).

In the study by Soltan Dallal *et al.*, 5(2%) isolates from pediatric diarrhea samples and 20 isolates (8%) from chicken meat samples were obtained from *Yersinia enterocolitica*. Biotyping of human *Yersinia enterocolitica* isolates identified 3 cases of biotype 1A, one case of biotype 1B, one case of biotype 2 and from chicken meat isolates, 16 isolates belonged to biotype 1A and 4 isolates belonged to biotype 1B. Presence of common pathogenic 1B and non-pathogenic 1A biotypes in pediatric diarrhea samples and chicken meat can indicate the cause of diarrhea in children (Soltan Dallal *et al.*, 2022).

Three strains of biotype 1A (75%) and one strain of biotype 1B (25%) which is pathogenic were found in this study. The human pathogenic serotype was not discovered during the serotype investigation, and it was assumed that the discovered strain most likely belonged to an environmental or animal pathogenic species. Le Guern *et al* found that the majority of *Yersinia* isolates in foods containing poultry meat, vegetables, fish, eggs, and dairy products were from environmental and non-pathogenic species, *Yersinia intermedia* (17.2%) came in second place to YE biotype 1A species (59.7%) (Le Guern *et al.*, 2016). By comparing the results of the previous studies with those of this study, it can be concluded that the discovery of *Yersinia* spp. in packaged salads supports the idea that

such food products are conducive to the continued growth of bacteria and that the strains isolated from the ingredients are responsible for this. The majority of the food contains biotype 1A of YE, indicating its widespread distribution in nature and the colonization of this strain in humans and asymptomatic animals' intestines.

The findings of the above-mentioned studies were consistent with each other and those of this study. This study also investigated the relationship between *Yersinia* spp. abundance and the type of protein material (chicken meat vs. sausage). According to the findings, the chicken meat Olivier salad prepared traditionally contained all *Yersinia* isolates, whereas the sausage Olivier salad (prepared either industrial or traditional) contained no *Yersinia* spp.

Soderqvist *et al.* investigated cold salad specimens containing chicken, ham, and vegetables for the presence of pathogens. YE, *Escherichia coli*, and *Listeria monocytogenes* pathogens were identified in these specimens, which were produced traditionally in retail centers. They also addressed the role of traditional cold food preparation and production centers in increasing the microbial load (pathogenic and non-pathogenic) in mixed salads (Söderqvist *et al.*, 2016). When the results of their studies were compared to those of this study, it can be observed that YE spp., like other pathogens, can contaminate the ingredients of Olivier salad both directly and indirectly as a result of inadequately cooking the raw ingredients and the lack of a potent thermal process before consumption. Due to the absence of preservatives in protein tissue, the high concentration of other nutrients, the ideal pH for growth, the high water activity, and the cold temperature of traditionally prepared Olivier salad, and bacteria are preserved in such foods and grow and spread easily under such conditions. As previously stated, YE is classified into six biotypes (1A, 1B, 2, 3, 4, and 5) based on biochemical reactions and 70 serotypes, of which only eleven have been associated with disease in humans, with the majority of cases involving just four virulent

serotypes: O:8 (biotype 1B), O:3 (biotype 4), O:9 (biotype 2), and O:5,27 (biotypes 2 and 3) based on the structure of the O antigen.

Regarding antimicrobial resistance, a similar study in Tehran found that YE isolates had the highest resistance to cephalothin and ampicillin (Soltan Dallal *et al.*, 2016, Soltan Dallal *et al.*, 2017). Another study in Switzerland showed that YE isolates had the highest resistance to ampicillin, cephalothin, and amoxicillin/clavulanate (Terentjeva and Bērziņš, 2010). The results of these studies were also consistent with the findings of this study.

Conclusion

This research examined the occurrence *Yersinia* bacteria in Olivier salad, a widely consumed culinary item. The results of the investigation indicated that traditional Olivier salad prepared with poultry meat often harbored *Yersinia* spp. The presence of preservatives in sausage-based specimens may account for the lower contamination of meat-based Olivier salad. Serotype tests, however, revealed that *Yersinia* biotypes had no human origin. It can be hence concluded that improper preparation of Olivier salad, particularly in processes related to cooking and preparing meat, is the root cause of *Yersinia* contamination in Olivier salad. However, the presence of highly pathogenic, antibiotic-resistant biotypes in this study raises alarming questions about the safety of ready-to-eat foods like Olivier salad and other similar products. As a result, this study recommends increasing food safety inspections and educating local and traditional food providers in order to prevent bacterial contamination and improve food quality.

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Conflict of interest

The authors declared no conflict of interests.

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Authors' contributions

All the author contributed to all parts of this manuscript.

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